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RESEARCH ARTICLE

Detecting Prion Protein Gene Mutations by Denaturing Gradient Gel Electrophoresis

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Mutations of the prion protein (PrP) gene are present in patients with Gerstmann–Sträussler–Scheinker syndrome (GSS), familial Creutzfeldt–Jakob disease (CJD), and fatal familial insomnia (FFI). We developed a denaturing gradient gel electrophoresis (DGGE) strategy that readily identifies point mutations in the PrP coding sequence. By comparison with appropriate controls, haplotypes often may be deduced. This method permits samples from many patients with GSS, CJD, as well as patients with unusual degenerative neurologic disorders, to be screened rapidly, sensitively, and inexpensively for the presence of known and novel PrP mutations. We illustrate the sensitivity of this approach by reporting 2 novel polymorphisms in the PrP coding sequence. © 1994 Wiley-Liss, Inc.

KEY WORDS: **Prion protein, PrP, Denaturing gradient gel electrophoresis, Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome**

INTRODUCTION

Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler–Scheinker syndrome (GSS) are etiologically related but phenotypically dissimilar neurologic disorders. GSS is characterized by slowly progressive ataxia, parkinsonism, and dementia beginning in the third through seventh decades and progresses to death over 2 to 10 years (Gerstmann et al., 1936; Masters et al., 1981). CJD is characterized by rapidly progressive dementia later associated with a variety of other neurologic signs including jerking of the extremities (myoclonus), muscle twitching (fasciculations), spasticity, and blindness (Kirschbaum, 1968; Roos et al., 1973; Brown and Gajdusek, 1991) Neuropathologic features of these conditions include loss and degeneration of neurons, gliosis, spongiform appearance of gray matter, and variable deposition of extracellular amyloid (Beck and Daniel, 1987). These amyloid deposits contain large amounts of prion protein (PrP) encoded by the PrP gene on chromosome 20 (Kitamoto et al., 1986; Sparkes et al., 1986). GSS is uniformly transmitted through autosomal dominant inheritance (Masters et al., 1981; Hsiao and Prusiner, 1990). In contrast, the majority of CJD patients have a sporadic condition with only 5 to 15% of CJD patients acquiring the disorder through autosomal dominant inheritance (Brown et al., 1987; Masters et al., 1979). Iatrogenic CJD is transmissible through organ transplants and pharmaceutical preparations containing material derived from CJD or GSS patients (Duffy et al., 1974; Tinter et al., 1986). There is compelling evidence that the transmissible agent or prion is composed largely, if not entirely, of an abnormal form of PrP designated PrP^{Sc} (Prusiner, 1982, 1987).

PrP coding sequence mutations are present in GSS patients and familial CJD patients (Fig. 1A) (Owen et al., 1989, 1992; Hsiao et al., 1989; Doh-ura et al., 1989; Fink et al., 1991; Nieto et al., 1991; Dhouhy et al., 1992; Hsiao et al., 1992; Goldgaber et al., 1989). These mutations are associated specifically with these disorders and the rare condition, fatal familial insomnia (FFI) (Lugaresi et al., 1991; Medori et al., 1991). When tested, these mutations have shown genetic linkage with these disorders (Hsiao et al., 1989; Dhouhy et al., 1992; Speer et al., 1991; Petersen

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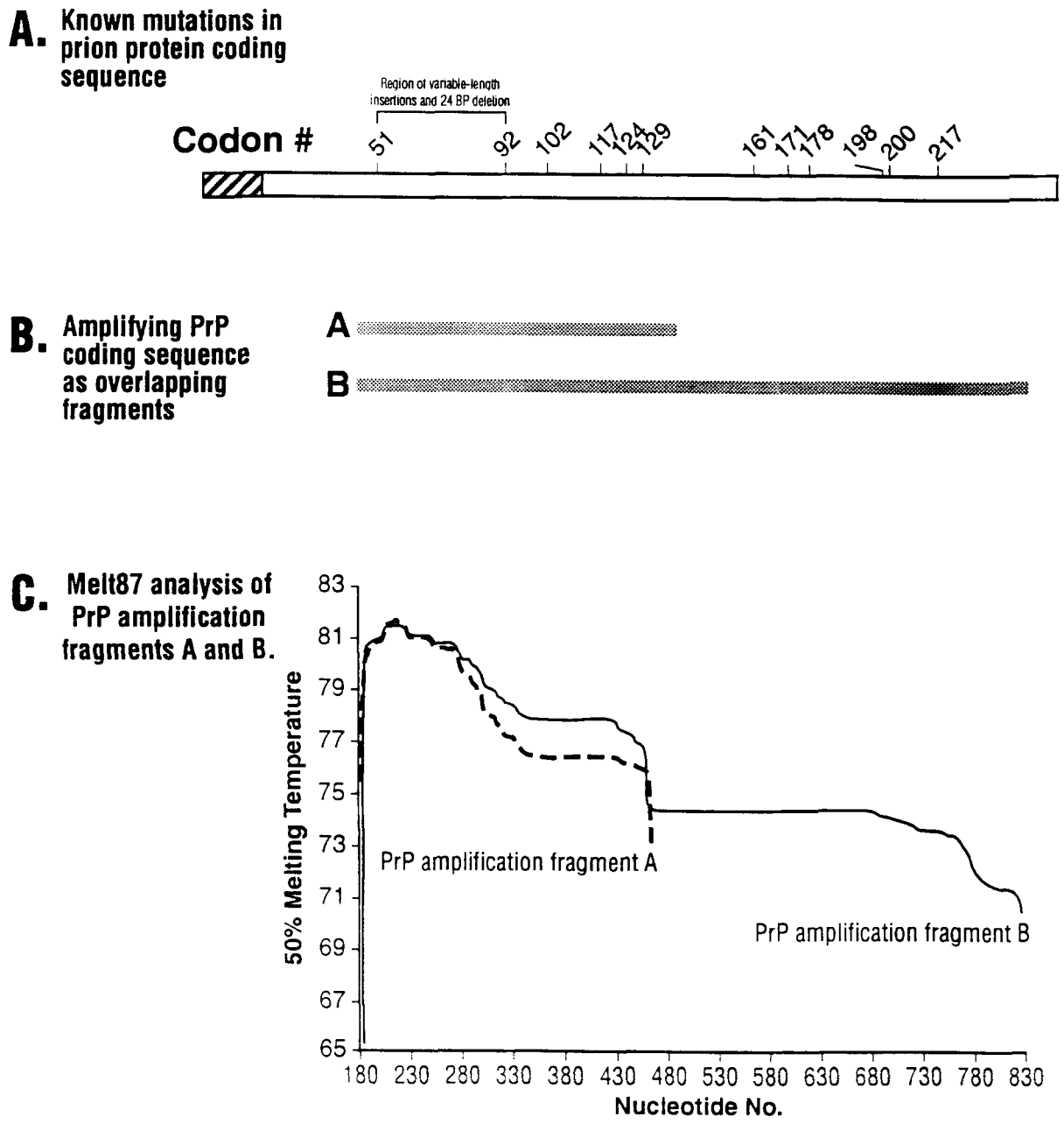

FIGURE 1. (A) The locations of known point mutations of the PrP coding sequence are shown: coding sequence (BP 50–808), open box; noncoding sequence (BP 1–49), hatched box. (B) Amplification fragments used for DGGE analysis. PCR primers and reaction conditions are given in Table 1. (C) Melt87 analysis of PrP amplification fragments A (—·—) and B (——).

et al., 1992). PrP mutation at codon 102^{Leu} was shown to be directly pathogenic when its expression produced an inherited neurodegenerative disorder in transgenic mice (Hsiao et al., 1990).

In general, PrP mutations associated with the syndrome of familial CJD are different from those associated with GSS (Goldfarb et al., 1990). Point mutations in PrP codons 102^{Leu}, 117^{Val}, 198^{Ser}, and 217^{Arg} are associated with GSS. An additional point mutation at codon 105^{Leu} was recently identified in GSS patients with spastic paraparesis (Kitamoto et al., 1993). PrP mutations associated

with familial CJD include insertions of varying numbers of a 24-base pair repeat (144 nucleotides, for example) (Owen et al., 1989, 1992) between codons 51 and 92 and point mutations in codons 178^{Asp} and 200^{Lys}. In addition to these disease-specific mutations, benign PrP polymorphisms have been found in normal subjects, including point mutations in codons 124 (Hsiao et al., 1992) and 117 and a 24-base pair deletion (Vnencak-Jones et al., 1992; Laplanche et al., 1990).

The polymorphism at PrP codon 129 may be clinically significant. The greatly increased frequency of homozygosity at codon 129 among sporadic CJD patients (approximately 90%) compared to normal subjects (63%) raises the possibility that this codon may contribute to the development of developing sporadic CJD (Palmer et al., 1991). Furthermore, the codon 129 genotype may affect the age of onset of familial CJD or GSS patients (Baker et al., 1991; Dhouhy et al., 1992) or whether patients exhibit the CJD phenotype or FFI phenotype (Goldfarb et al., 1992).

Observations of PrP mutations in GSS patients with absent or atypical (Collinge et al., 1990; Dhouhy et al., 1992; Hsiao et al., 1992) neuropathologic findings have raised the possibility that PrP-related disorders may be underrecognized. It is possible that some patients with inherited, degenerative neurologic syndromes of undetermined etiology have PrP mutations. Comprehensive evaluation of the PrP gene in these patients requires more than simply screening for the presence of known PrP mutations. Previously, one of us (J.F.) used denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman, 1983) and allele-specific DNA sequencing to identify a novel PrP mutation (Fink et al., 1991) in a familial CJD patient. This approach has been extended to allow mutations in other regions of the PrP coding sequence to be detected. We show that this technique readily identifies known PrP point mutations. In addition, this method facilitates detection of novel PrP mutations in samples from many patients. We illustrate the sensitivity of this approach by reporting two novel polymorphisms of the PrP coding sequence.

MATERIALS AND METHODS

Patients and DNA Samples

Genomic DNA samples were obtained from CJD and GSS patients for whom PrP mutations had been identified by restriction enzyme and DNA sequence analysis. The exceptions were the normal subject for whom the codon $129^{Val}/129^{Val}$, codon $129^{Met/Met}$, and codon $129^{Met/Val}$ genotypes were demonstrated by Nsp1 restriction enzyme analysis (data not shown).

Computer Analysis

The MELT87 computer program (generously provided by L. Lerman, Massachusetts Institute of Technology) was used to design overlapping amplification fragments in which regions of the PrP coding sequence known to have point mutations (nucleotides 353 to 698) would occur in the "lowest melting domain."

PrP Amplification

The PrP coding sequence was amplified as overlapping fragments (Fig. 1B) from genomic DNA extracted from leukocytes. The only exceptions were DNA samples containing codon 117^{Ala} and 117^{Val} for which the PrP alleles cloned into bacterial plasmids served as the PCR template. PCR primers and amplification parameters are given in Table 1. Nucleotide positions are numbered according to Kretzschmar et al. (1986).

Denaturing Gradient Gel Electrophoresis

Following amplification, PrP fragments were denatured by heating to 95°C for 10 min. Reannealing was carried out at 80°C for 2.5 min followed by 42°C for 5 min. 5 ml of loading buffer (40% glycerol, 0.1% bromphenol blue) were added to the 50 ml PCR product. Aliquots (20–25 μl) of this mixture were electrophoresed on denaturing gradient polyacrylamide gels as specified in Table 1. Mutant and normal PrP amplified from plasmid templates were mixed (1:1) prior to denaturation and reannealing in order to mimic the situation of amplifying genomic DNA from a patient who was heterozygous at codon $117^{Val}/117^{Ala}$.

Restriction Enzyme Analysis

For samples containing novel DGGE polymorphisms, a portion of the prion gene (bp 361 through 607) was amplified [5′ primer, GCCAAAAACCAACATGAAGC; 3′ primer, TGGTTGTGGTGACCGTGTGC; 2 mM $MgCl_2$; 35 cycles of 58°C (45 sec), 72°C (1.25 min), 94°C (1 min)]. Amplification products were electrophoresed on a low-melting agarose gel and purified by Magic Prep columns (Promega Corp). DNA samples were separately digested in a total volume of 20 ml with 10 units of AccI and *Bst*71I for 2 hr at 37°C (AccI) or 50°C (*Bst*71I). Following digestion with *Bst*71I, proteinase K (10 mg/ml) was added to remove excess *Bst*71I. Aliquots (10–20

TABLE 1. PCR Primers and Amplification Parameters

Fragment A (Effectively scans PrP nucleotides 270 to 448)

PCR Primers: "5A": CTGGAGGCAACCGCTACCCA (PrP nucleotides 180 → 199); "3A": TGTATGATGGGCCTGCTCAT (PrP nucleotides 468 → 449)

PCR: Each 50 ml reaction contains 1 mg template DNA, 200 mM each dNTP, 0.25 unit *Taq* polymerase, 0.66 mg of each primer, 2 mM $MgCl_2$; 94°C, 1.0 min; 60°C, 0.5 min; 72°C, 1.25 min; 35 cycles

DGGE: Stacking gel: 6% polyacrylamide gel with 29:1 crosslinker; gradient gel: 9% polyacrylamide gel with 22:1 crosslinker; gradient of 50 to 75% denaturant (100% denaturant = 40% formamide/7 M urea); buffer: 40 mM Tris-acetate/1 mM EDTA, pH 7.8; electrophorese at 14 mA, 65°C, for 18 hr

Fragment B (Effectively scans PrP nucleotides 440 to 787)

PCR Primers: "5A": (sequence given above) "3B": CCTTCCTCATCCCACTATCAGGAAGATGAG (PrP nucleotides 817 → 788)

PCR: Reaction conditions are the same as for fragment A; 94°C, 1.0 min; 60°C, 0.75 min; 72°C, 1.5 min; 35 cycles

DGGE: Stacking gel: 5% polyacrylamide gel with 29:1 crosslinker; gradient gel: 5% polyacrylamide gel with 29:1 crosslinker; gradient of 40 to 75% denaturant (100% denaturant = 40% formamide/7 M urea); buffer as above; electrophorese at 28 mA, 60°C, for 16 hr

ml) of each sample were electrophoresed on 1.6% agarose gels.

RESULTS

We used the MELT87 computer program (Lerman 1990) (Fig. 1C) to design a strategy in which regions of the PrP coding sequence known to have point mutations would be in the lowest melting domain of an amplification fragment. Figure 2A and B show that known PrP point mutations are readily detectable by DGGE.

The sensitivity of this approach suggested that novel mutations in these regions would be readily detected. This prediction was confirmed by finding unique DGGE polymorphisms in a control subject and in a patient with Alzheimer's disease (AD) (Fig. 3A). The control subject was a male who died at age 69 and had no evidence of AD or spongiform degeneration at postmortem examination. Direct DNA sequencing revealed substitution of guanine (for adenine) at nucleotide 561 in the control subject. This polymorphism creates a *Bst*71I site (Fig. 3B) and predicts substitution of serine for asparagine at codon 171 (AAC → AGC). Whether the codon 171^{Ser} → Asp is a rare but benign variant or potentially pathogenic is not known. Direct DNA sequencing of the AD patient's sample revealed substitution of adenine for guanine at nucleotide 532. This polymorphism creates an *Acc*I site, deletes an *Rsa*I site (Fig. 3B), and does not change the valine at codon 161 (GTG → GTA).

In addition to detecting known and novel PrP mutations, when used in conjunction with restriction enzyme analysis, our method often can determine PrP haplotypes. Determination of PrP haplotypes by DGGE is particularly useful in cases where the mutation either does not change a restriction site or when the location of the novel restriction site makes it difficult to detect the resulting RFLP (such as in the case of codon 129 and 124 polymorphisms). Lanes 1, 2, 4, and 5 in Figure 2A illustrate how haplotypes may be deduced and the effects of mutations on the relative location of homoduplexes. Consider as controls samples in Figure 2A, lanes 1 and 2 amplified from normal subjects who were homozygous at codon $129^{Met/Met}$ (lane 1) and homozygous at codon $129^{Val/Val}$ (lane 2). Lane 4 contains PrP amplified from a normal subject heterozygous for a benign, single nucleotide change at codon 117^{Ala} → Ala (GCA → GCG). Previously, digesting this patient's sample with *Nsp*I indicated that the subject was heterozygous at codon $129^{Met/Val}$. The upper homoduplex has the same mobility as the normal subject with codon $129^{Met/Met}$ (lane 1), indicating that the subject's normal allele (117^{GCA}) encodes 129^{Met}. Knowing that the subject is heterozygous at both codons 117(GCA → GCG) and $129^{Met/Val}$ indicates that the other allele must encode 117^{GCG} and 129^{Met}. The haplotypes are deduced to be $117^{WILD\ TYPE}$ (GCA), $129^{Met}/117^{VARIANT}$ (GCG), 129^{Val}. The lower (mutant) homoduplex focuses slightly below that of the normal 129^{Val}-containing homoduplex in lane 2 owing to the slightly increased melting temperature conferred by the A to G nucleotide substitution. This illustrates the correlation between the nature of the polymorphism and the relative location of the mutant homoduplex. G (or C) to A (or T) changes decrease the melting temperature of the mutant homoduplex causing it to focus earlier (higher) in the gel. The converse is true regarding A (or T) to G (or C) changes. Lane 5 (Fig. 2A) contains material from a GSS patient with a pathogenic double mutation at codon $117^{GCA \rightarrow GTG\ (Ala \rightarrow Val)}$. Like the

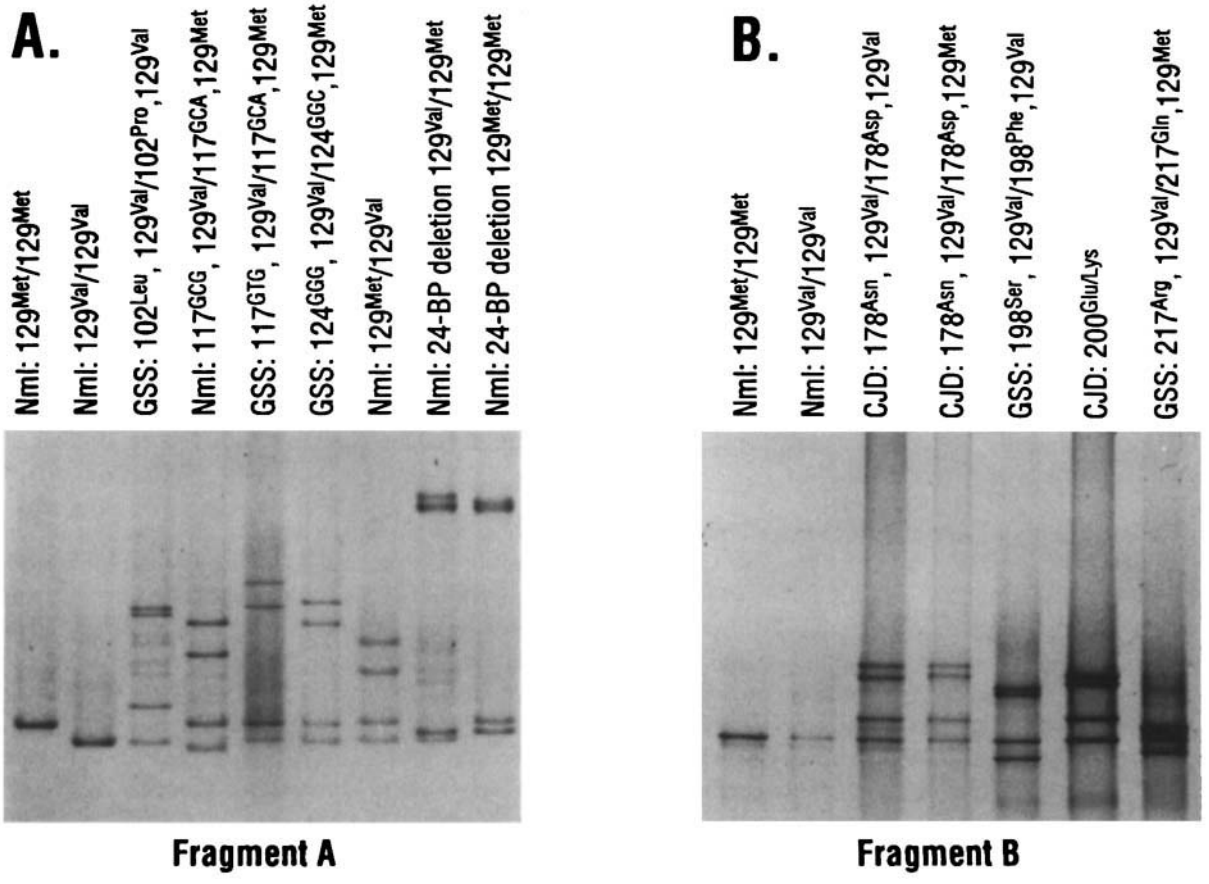

FIGURE 2. **Demonstration of PrP point mutations by DGGE analysis. (A) DGGE of PrP Fragment A amplified from a normal subjuct homozygous at codon $129^{Met/Met}$ (lane 1); a normal subject homozygous at codon $129^{Val/Val}$ (lane 2); a GSS patient heterozygous for a pathogenic mutation at codon $102^{Leu/Pro}$ and a polymorphism at codon $129^{Val/Val}$ (lane 3); a normal subject with a benign, single-nucleotide change at codon $117^{GCA\rightarrow GCG}$ and heterozygosity at codon $129^{Met/Val}$ (lane 4); a CJD patient heterozygous for a pathogenic, double nucleotide mutation at codon $117^{GCA\rightarrow GTG}$ (Ala→Val) and heterozygous for a benign polymorphism at codon $129^{Met/Val}$ (lane 5); a normal subject heterozygous for a benign polymorphism at codon $129^{Met/Val}$ (lane 6); a normal subject with a 24 base pair deletion who was also heterozygous at codon $129^{Val/Val}$ (lane 7); a normal subject with a 24 base pair deletion who was homozygous at codon $129^{Met/Met}$ (lane 8). (B) DGGE of PrP Fragment B amplified from a normal subject homozygous at codon $129^{Met/Met}$ (lane 1); a normal subject homozygous at codon $129^{Val/Val}$ (lane 2); a familial CJD patient heterozygous at codon $178^{Asp/Asn}$ who was homozygous at codon $129^{Val/Val}$ (lane 3); a familial CJD patient who was heterozygous at codon $178^{Asp/Asn}$ and heterozygous at codon $129^{Met/Val}$ (lane 4); a GSS patient heterozygous at codon $198^{Phe/Ser}$ and homozygous at codon $129^{Val/Val}$ (lane 5); a CJD patient heterozygous at codon $200^{Glu/Lys}$ (lane 6); a GSS patient heterozygous at codon $217^{Gln/Arg}$ and heterozygous at codon $129^{Met/Val}$ (lane 7).**

sample in lane 4, this subject also has the pathogenic codon 117^{GCA} mutation on the same allele containing the benign change at codon 129^{Val}. In contrast to the downward displacement of 117^{GCG}, 129^{Val} (compared with 117^{GTA}, 129^{Val}), the double nucleotide substitution causes negligible displacement of the homoduplex since the two substitutions have opposing affects on melting temperature (C → T and A → G). Note, however, the separation of homoduplexes from heteroduplexes. Compared to homoduplexes, the heteroduplexes have greatly reduced melting temperature because of the 2-base pair mismatch that forms when these mutant and normal alleles reanneal (Fig. 2A, lanes 4 and 5).

As expected, the largest separation of homo- and heteroduplexes occurred in samples with the 24-base pair deletion (Fig. 2A, lanes 8 and 9). Since this deletion occurs in the octapeptide repeat region, heteroduplexes may form in 2 ways. The deletion creates a 24-base pair mismatch that may be placed either internally (creating a 24-base pair loop) or at the 5′ end (creating staggered ends and a terminal region of nonhomology). Owing to slight variations in this repeat region, however, this latter structure creates 3 separate single-nucle-

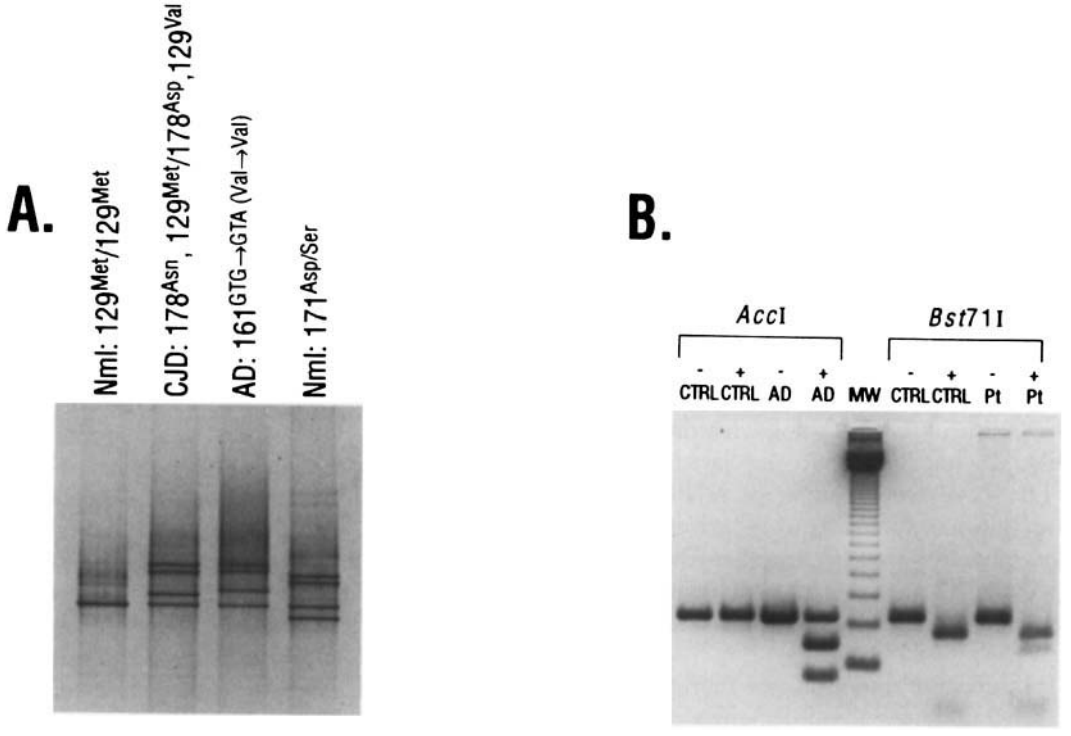

FIGURE 3. Detection of novel PrP point mutations. (**A**) DGGE detection of novel polymorphisms in PrP coding sequence. Control samples in lanes 1 and 2 are PrP (fragment B) amplified from a normal subject homozygous at codon $129^{Met/Met}$ (lane 1) and a familial CJD patient heterozygous for a benign polymorphism at codon $129^{Met/Val}$ and heterozygous for a pathogenic mutation at codon $178^{Asp/Asn}$ (lane 2). Samples with novel DGGE polymorphisms (lanes 3 and 4) are from an Alzheimer's patient homozygous at codon $129^{Met/Met}$ and heterozygous for a benign nucleotide polymorphism at codon $161^{GTG\rightarrow GTA\ (Val\rightarrow Val)}$ (lane 3); and a control subject homozygous at codon $129^{Val/Val}$ and heterozygous at codon $171^{AAC\rightarrow AGC\ (Asp\rightarrow Ser)}$ (lane 4). (**B**) Restriction enzyme analysis of PrP amplification fragment B confirms the presence of novel polymorphisms. PrP amplified from control subject (Ctrl) (lanes 1 and 2) and Alzheimer's patient (AD) with codon 161 polymorphism (lanes 3 and 4) prior to (−) and following (+) digestion with *Acc*I illustrate the creation of a novel *Acc*I site in the AD patient (lane 4). PrP amplified from control subject (Ctrl) (lanes 6 and 7) and subject (Pt) with codon 171 polymorphism (lanes 8 and 9) prior to (−) and following (+) digestion with *Bst*71I illustrate the creation of a novel *Bst*71I site in the lane 9. Lane 5 contains molecular weight (MW) marker (123 bp ladder).

otide mismatches (at nucleotides 277, 295, and 301). We feel that this latter heteroduplex with staggered 5′ ends and 3 separate single-nucleotide mismatches best explains the large difference in melting profiles (and resulting gel separation) between hetero- and homoduplex species with the 24-base pair deletion.

DISCUSSION

DGGE is an extremely sensitive and efficient method for screening large numbers of samples for single nucleotide substitutions, small insertions, and deletions. Principles of mutation detection by DGGE are reviewed elsewhere (Meyers et al., 1987).

Known PrP point mutations (reviewed in Palmer and Collinge, 1993) occur between nucleotides 353 and 698, and are clearly evident in our PrP amplification fragments. We have found two novel PrP point mutations using our method. This experience predicts that DGGE analysis of PrP fragments A and B can identify other, currently unknown mutations in the region of the PrP gene between nucleotides 270 and 787. With the use of an alternate 3′ primer for fragment B (5′ → 3′: GTGAAAACAGGAAGACCTTCC), it should be possible to extend the "scannable" region to include the remaining 19 base pairs in the 3′ end. There are no known point mutations in nucleotides 1 through 270 of the PrP coding sequence (the first 49 nucleotides of which are not translated). All known polymorphisms in this region are either insertions of varying numbers of 24 nucleotide repeats or a 24-base pair deletion and are readily detectable by PCR and conventional gel electrophoresis. Alternatively, temperature gradient gel electrophoresis (TGGE) (Riesner et al., 1992) or single-strand conformation analysis

(Orita et al., 1989) may be useful to analyze this region.

It is possible that there are additional PrP mutations, particularly in familial CJD patients. It is not known whether familial CJD patients who lacked PrP codon 102^{Leu} or 200^{Lys} mutations (Goldgaber et al., 1989) had other PrP mutations (such as codon 178^{Asn}, for instance) or entirely normal PrP coding sequences. Finding PrP mutations in such patients would confirm the diagnosis and help provide genetic counseling for relatives at risk. The absence of PrP mutations in such patients would raise the possibility that these patients with clinical and pathologic features similar to familial CJD had genetically unrelated disorders or that PrP involvement might be related to abnormal amounts of PrP (due to increased rates of transcription, translation, increased mRNA stability, or reduced PrP catabolism, for instance), or even aberrant posttranslational modification of PrP.

A PrP insertional mutation was found in a patient with familial ataxia and dementia characteristic of GSS in whom there was no evidence of spongiform degeneration (Collinge et al., 1990; Poulter et al., 1992). Amyloid deposits in this patient were evident only with immunohistochemical staining (Poulter et al., 1992). Furthermore, mutations in PrP codons 198^{Ser} and 217^{Arg} were present in patients with dominantly inherited dementia, ataxia, and parkinsonism whose postmortem findings of senile plaques and neurofibrillary tangles were similar in appearance (but not distribution) to Alzheimer's disease in contrast to spongiform degeneration characteristic of GSS and CJD (Dhouhy et al., 1992; Hsiao et al., 1992). Using the parameters of disease transmissibility (Brown et al., 1993) and immunologic detection of PrP (Brown et al., 1993; Kitamoto et al., 1992), Brown (1993) conclude that transmissible spongiform encephalopathies are quite rare and not likely to be misdiagnosed as other neurodegenerative disorders. Nonetheless, we should consider the possibility that all disorders related to prion gene mutations may not exhibit a uniformly high rate of disease transmission to laboratory animals or a uniform neuropathologic pattern. The first category of patients to be examined for *novel* PrP mutations include familial CJD patients lacking known PrP mutations as well as some patients diagnosed as early-onset Alzheimer's disease. Some early-onset familial AD patients, and particularly those with cortical Lewy bodies, have clinical and pathologic features similar to CJD including onset of symptoms in the third or fourth decade, subacute progressive dementia, myoclonus, and spongiform degeneration (Feldman et al., 1963; Hansen et al., 1990). Although previous efforts have failed to detect PrP insertional mutations and point mutations at codons 102^{Leu}, 117^{Val}, and 200^{Lys} in AD patients (Schellenburg et al., 1991), the possibility of novel PrP mutations in patients with atypical dementia has not been systematically evaluated. The DGGE approach allows investigators to rapidly determine whether PrP mutations are present in seemingly unrelated neurodegenerative disorders, including progressive subcortical gliosis, progressive myoclonic epilepsy, olivopontocerebellar atrophy, multisystem atrophy, progressive supranuclear palsy, and spinocerebellar degenerations.

Familial CJD and GSS seem to be useful models for more common inherited neurodegenerative disorders. Elucidating the central role of abnormal PrP in GSS and familial CJD and creating an animal model of GSS have advanced our knowledge of mechanisms by which mutations can cause inherited brain disease. Defining the nature and extent of additional PrP mutations in patients with disparate neurologic conditions will provide further insight into the basis for regionally specific neuronal degeneration in these conditions. Unraveling this pathophysiologic cascade may give some insight into potential mechanisms underlying the more prevalent degenerative neurologic disorders of unknown etiology.

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